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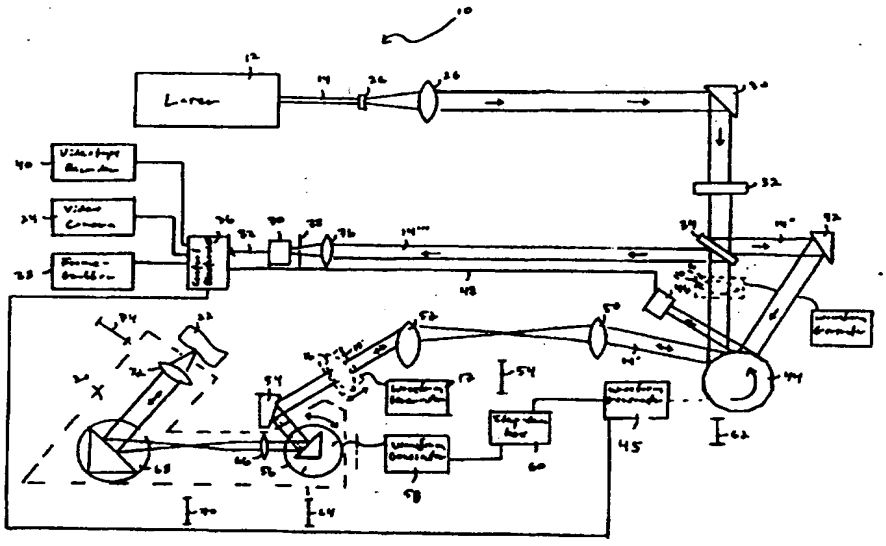

(57) Abstract

A confocal microscope (10) for generating a video-rate, real-time image of a sample (22). The microscope (10) includes an optical delivery system, and horizontal (44) and vertical (56) scanning elements for generating a two-dimensional imaging field. The confocal microscope (10) additionally includes a focal-plane scanning element (16), positioned along the imaging fields' optical pathway, which includes an oscillating, motor-driven reflecting or transmitting optical component for spatially varying, in a time-dependent manner, the focal plane of the two-dimensional imaging field. Alternatively, in another aspect the microscope (10) features a pivoting articulated arm (20) for housing the vertical scanning element (56) and the imaging lens (72). The arm (20) includes at least one reflective elbow joint (68) configured to receive the two-dimensional imaging field from the vertical scanning element (56) and then deliver the field to the imaging lens (72).

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"VIDEO-RATE CONFOCAL SCANNING LASER MICROSCOPE"

Background

The invention relates to confocal microscopes.

In general, confocal microscopy involves focusing and scanning light through a sample plane in order to form a high-resolution, highly magnified image. Conventional confocal microscopes image samples by generating a series of thin "optical sections" which are high-resolution images of thin planes within the sample (see, e.g., U.S. Patents 4,827,125; 4,198,571, the contents of which are incorporated herein by reference). Confocal microscopy, in general, is done *in vitro*.

There are two conventional types of confocal scanning microscopes. The tandem scanning confocal microscope ("TSCM"), employs a rotating pinhole disk illuminated by a light source such as a mercury lamp. During operation, the disk sweeps a focal spot through a sample, and is additionally used to spatially filter back-scattered light. This instrument has been used, for example, to image sections of skin. A similar device, the confocal laser scanning microscope ("CLSM"), uses a laser beam to image a sample, such as a biopsied tissue sample. In this instrument, the laser beam is focussed to a nearly diffraction-limited spot within a single plane of the sample. The spot is then scanned across the plane, or alternatively, the sample is translated using a micrometer stage. In general, the CSLM has greater detection power, superior wavelength selectivity, and better illumination power than the TSCM.

Summary

In general, the invention features a confocal microscope for generating a video-rate, real-time image of a sample. The microscope includes an optical delivery system including a light source for generating an imaging field. This field is modulated using a horizontal scanning element including a motor-driven, movable reflecting component positioned to receive and then reflect the imaging field in a time-dependent manner to generate an elongated horizontal imaging field along a horizontal axis. A vertical scanning element, including a motor-driven reflecting component which angularly oscillates about an axis in an adjustable, time-dependent manner, is positioned to receive and then reflect the horizontal imaging field along a vertical axis, thereby generating a two-dimensional imaging field including both horizontal and vertical imaging fields. This field is focused to a focal plane in the sample using an imaging lens; the lens is also used to receive the remitted (i.e., reflected, scattered, fluoresced, or emitted in some other way) light. Detector means are then used for converting the remitted optical image into a series of electrical signals, and image processing means are used to process the signals to generate a real-time, video-rate image of the sample.

In one particular aspect, the confocal microscope features a focal-plane scanning element, positioned along the imaging field's optical pathway, which includes an oscillating, motor-driven reflecting or transmitting optical component for spatially varying, in a time-dependent manner, the focal plane of the two-dimensional imaging field. Alternatively, in another aspect, the microscope features a pivoting articulated arm for housing the vertical scanning element and the imaging lens. The arm includes at least one reflective elbow

joint configured to receive the two-dimensional imaging field from the vertical scanning element and then deliver the field to the imaging lens. Alternatively, the arm may contain two or more pivoting points. In another aspect, the microscope features both the focal-plane scanning element and the articulated arm.

In preferred embodiments, the focal-plane scanning element is mounted on an oscillating stage configured to translate with an adjustable longitudinal displacement along the optical pathway. Preferably, the focal-plane scanning element (e.g., a lens) is positioned to receive and then transmit a two-dimensional imaging field which includes both horizontal and vertical imaging fields.

The confocal microscope also preferably includes at least one controller which contains a frequency source for selectively varying the magnitudes of the angular rotation and longitudinal displacement of, respectively, the vertical and focal-plane scanning elements; most preferably, these elements are driven by a common frequency source at a frequency of between 25 and 100 Hz.

In another embodiment, the vertical scanning element is positioned at a pivot point of the articulated arm, which further includes a focussing lens positioned along the two-dimensional field's optical pathway between the vertical scanning element and the imaging lens. The focussing lens is preferably configured to image the two-dimensional field onto an entrance aperture of the imaging lens so that spatial displacements of the field at the aperture are minimized.

The light source of the confocal microscope is preferably a laser which emits radiation in the infrared spectral region, preferably between about 1550 nm and 1800 nm, less than 1400 nm, and most preferably at about 1600 nm. The laser, for example, may be a Nd:YAG laser. Alternatively, laser is a semiconductor diode laser.

Infrared light is desirable as the radiation deeply penetrates living tissue, and can thus be used to form more complete images.

In another aspect, the invention provides a method of generating a video-rate, real-time *in vivo* image of living tissue. The method includes the steps of a) illuminating the tissue with an axially translated optical field which is focussed through a pivoting articulated arm attached to a microscope objective lens; and b) scanning the optical field through the tissue, which, following irradiation, remits light through the lens and arm to a photodetector and a video monitor. Both the photodetector and video monitor are operably linked, and together form a video-rate, real-time *in vivo* image of the tissue.

In preferred embodiments, the optical field is scanned along the x and y dimension, the tissue is human skin, and melanin is used as a naturally occurring contrasting agent.

The inventions have many advantages. In particular, the confocal microscope described herein allows real-time, video-rate imaging of tissue *in vivo*. This allows the operator to view optical sections of living tissue which are oriented along horizontal, vertical, or variably angled planes. In this manner, high-resolution three-dimensional images can be non-invasively constructed, thereby providing an alternative to conventional histopathology. This allows the patient to be spared a the pain, infection risk, scarring and extra cost associated with standard tissue biopsies.

In addition, by using near-infrared or infrared wavelengths, the confocal microscope allows images to be taken from well below the surface of the tissue; this advantage is particularly useful for imaging the layers of human skin. The confocal microscope's articulated arm

allows convenient positioning and contact with tissue. In this embodiment, the imaging lens contained the arm is placed in direct contact with the tissue, thereby minimizing any relative motion between the lens and the tissue. The feature additionally stabilizes the depth of the plane to be imaged. Other advantages of the invention will be evident from the following description and from the claims.

Brief Description of the Drawings

Fig. 1 is a top view of the confocal microscope according to the invention;

Fig. 2 is a top view of the articulated arm of the confocal microscope;

Figs. 3A-3C are, respectively a top view of the two-dimensional optical field generated by the polygon mirror and the galvanometric mirror, an expanded top view of two adjacent raster lines from the two-dimensional field, and an expanded top view of two overlapping raster lines from the field;

Figs. 4A-4C are side-sectional views illustrating, respectively, depth-dependent horizontal sections taken along the z axis, angularly adjustable views taken from the xz plane to the xy plane, and angularly adjustable views taken from the yz plane to the xy plane;

Fig. 5 is a schematic drawing of a vertical cross-section of the skin (left side) as the corresponding image taken along the horizontal plane using the confocal microscope (right side);

Figs. 6A-6H are confocal microscope images of, respectively, the stratum corneum, the granular, the upper spinous, the lower spinous, and basal cells on the dermal papillae (6E-6H);

Figs. 7A-7C are confocal microscope images of blood flowing through the papillary dermis;

Figs. 8A and 8B are confocal microscope images of, respectively, darkly pigmented and relatively lighter pigmented skin; and

Fig. 9 is an image taken of the skin using a confocal microscope employing an infrared (i.e., a Ti:Sapphire laser) light source.

Detailed Description

Referring first to Fig. 1, a confocal microscope 10 according to the invention includes a laser 12 for providing an irradiating optical field 14, a scanning element (such as a lens 16 mounted on a scanning platform 18) positioned along the optical axis (i.e, the z axis), and an articulated arm 20 which includes a series of optical elements configured to deliver the optical field 14 to the tissue 22 to be imaged. The microscope 10, in general, allows tissue 22 (e.g., human skin) to be imaged *in vivo* and in real time, with the resulting images being scanned at a rate suitable for viewing using conventional television and video equipment. During operation, the plane of imaging may be continuously adjusted between horizontal and vertical optical sections, thereby allowing the user to monitor a wide range of depth-dependent images, each having resolution on the cellular level.

Generation of the imaging field and collection of a resulting image, is, in general, performed using procedures commonly used with commercially available confocal microscopes. In some embodiments, the imaging field 14 emitted from the laser 12 typically has a beam diameter of the order of between 1 - 5 mm, and may be expanded using a two-lens telescope system including, for example, concave 26 and plano-convex 28 lenses. Prior to being modulated with the imaging optics of the microscope 10, the expanded beam may be reflected off a series of beam-steering optics, such as a mirror 30 coated to have

a high reflectivity at the central wavelength of the emitted optical field 14. While a single lens is shown, it is understood that more complex beam-steering optics, or, alternatively, fiber optic delivery systems (e.g., single mode fibers), may be used to deliver the imaging field to other components of the microscope.

An adjustable neutral density filter 32 allows selective variation over the intensity of the imaging field. Typically, a power level is chosen to maximize the intensity and contrast of the image, while sparing the tissue 22 from light-induced damage; continuous-wave power levels ranging from several milliwatts to a several watts may be used. Once an appropriate power level is chosen, the imaging field 14 is propagated through a partially transmitting beam-splitting optic 34 to generate a imaging field 14′ for generating the imaging signal, and a secondary field 14′′ for generating a light-induced scan pulse for input to the control electronics 36 which, in turn, are used to drive a standard video monitor 24. If necessary, video-storage devices, such as a frame-grabber 38 and videotape recorder 40, and also used to generate permanent images.

In order to generate the scan pulse, the secondary field 14′′ is first reflected off a second series of standard beam-steering optics, including a mirror 42, and onto a the reflecting surface of a rotating polygon mirror 44. This optical device includes multiple reflecting "facets" which reflect the secondary field onto an optical detector 46, such as a standard PIN photodiode. Typically, the polygon mirror has about 25 facets, and is driven by a standard rotating motor at about 40,000 r.p.m. (this frequency corresponds to 15.7 kHz, the U.S. standard video rate). Other combinations of rotational frequencies and polygon geometries may be used to generate signals at 15.7 kHz. For example,

galvanometric scanning devices may be used to replace the rotating polygon mirror.

The detector 46 is preferably a split diode having two separate, oppositely biased detecting regions (composed of, e.g., silicon). When the secondary field impinges the detector 46 so that both regions are illuminated, a bi-phasic electrical waveform, indicated as the signal line 48, results. The zero-crossing point of this signal is used to trigger the control electronics 36, which, are then used to generate the electrical scan pulse.

The imaging field 14' passing through the beam-splitting optic 34 has an intensity, for example, of about 70-90% of the incident field 14, and is reflected off the rotating polygon mirror and onto a pair of plano-convex lenses 50, 52 used for imaging. The first lens 50 typically has a focal length of between 50 and 100 mm, an f/2, and is displaced from the reflecting surface of the polygon mirror 44 by a distance equivalent to the lens's focal length. As the polygon mirror rotates, the reflected imaging field 14' is angularly displaced, or scanned, in an oscillatory fashion along a plane oriented horizontally relative to the optical or z axis. The scanned field then impinges different positions along the lens 50, where it is focused to a waist along a raster plane (indicated in the figure by the line 54). The second lens 52 of the imaging system typically has a focal length of between 15 and 20 cm, an f/5.3, and is displaced from the raster plane by a distance equivalent to its focal length in order to collimate the horizontally scanning field. Once collimated, the field may be reflected off a mirror 54, where it is delivered into the articulated arm 20 and used for imaging the tissue 22.

The articulated arm 20 features a galvanometric mirror 56, positioned at the pivot point of the arm, which oscillates back and fourth at a frequency and amplitude determined by a driver 58 (e.g., a waveform generator). Oscillation of the mirror in this fashion allows the field 14' to be additionally scanned along the y axis. The driver 58 is preferably electrically synchronized through a step-down box 60 with the driver 45 connected to the electronic control box 36, which is the source of the galvanometer driver signals. The rotating polygon mirror has its own crystal controller. The degree of rotation of the mirror 56 is determined by the amplitude of the driving periodic waveform. The galvanometric mirror 56, in addition, is positioned at a distance equivalent to the focal length of the second lens 52 included in the two-lens imaging system. In this configuration, the reflecting surface of the rotating polygon mirror forms the object plane of the imaging system, indicated in the figure by the line 62; the reflecting surface of the galvanometric mirror forms the optical conjugate, or image plane, indicated in the figure by the line 64. Arrangement of the optics 44, 50, 52, and 56 in this fashion minimizes the spatial displacement of the imaging field 14' relative to the surface of the galvanometric mirror during operation, thereby minimizing spatial wandering of the field. An additional lens 66, typically having a focal length of between 50 and 75 mm and an f/3, is displaced from the galvanometric mirror by a distance of between 100 and 200 mm, and is used to focus the field 14' prior to reflection off at least one reflecting "elbow" joint 68 contained in the articulated arm 20. Preferably the elbow joint angle is 90°.

The imaging field 14', following reflection from the elbow-joint mirror 68, impinges an imaging lens 72,

which is most preferably a microscope objective lens having a focal length of a few millimeters, e.g., up to about 1 millimeter. The microscope objective lens 72 is positioned relative to the lens 66 so that the plane corresponding to its entrance aperture, indicated in the figure by the line 74, forms the conjugate image plane relative to the plane defining the reflecting surface of the galvanometric mirror (line 64). As before, positioning of the microscope objective lens 72 in this manner allows its entrance aperture to form the object plane relative to the conjugate image plane. This allows the spatial wander of the scanned field (in the xy plane) to be minimized at the aperture, thereby minimizing non-uniformity in light intensity over the field of view on the sample.

The lens 72 focusses the imaging field to different depths in the tissue 22. In embodiments, the depth of focus may be adjusted by mechanically translating the lens 72 in a normal direction relative to the tissue surface. Alternatively, an additional focussing lens 16 (having, for example a focal length of between 100 cm and 2 m) mounted on the scanning platform 18 may be placed at any point along the optical axis, and translated along the beam path in an oscillatory manner in order to continuously vary, or scan along the optical or z axis, the depth of focus. Preferably, the scanning platform is driven by the same driver used to power the galvanometric mirror; alternatively, a separate driver may be used. The scanning lens 16 may optionally be positioned at other points in the beam path, such as prior to the mirror 30 as indicated in the figure. In combination with the x and y scanning initiated by, respectively, the polygon and galvanometric mirror, scanning an optic along the optical (z) axis allows real-time, three-dimensional imaging of horizontal and

vertical or tilted sections of tissue in a continuously adjustable manner.

Actual imaging is preferably performed using light remitted (i.e., reflected or emitted light) from the sample. For example, a small portion 14''' of the imaging field 14' may be reflected back off different components of tissue, such as cells, following irradiation with the imaging field. Only light reflected from tissue components lying at the imaging lens's focal plane will remain collimated following propagation through the system of optics; light reflected from other planes in the tissue will be spatially divergent or convergent. After reflecting off the polygon mirror 44, the collimated reflected field impinges and is reflected off of the beam-splitting optic, and is focussed by a lens 76 through an aperture 78 and onto the face of an optical detector 80, such as an avalanche photodiode. The size of the aperture is chosen so that only light reflected from the focal plane in the sample is passed; typically the aperture has a diameter of between 25 and 100 microns.

Light irradiating the optical detector 80 generates a light-induced signal, indicated in the figure by the signal line 82: horizontal components of the reflected field 14''' generate electrical signals at the standard video frequency of 15.7 kHz, while vertical components of the signal generate electrical signals at the lower frequency of about 60 Hz, a rate corresponding to the frame-renewal frequency of conventional video equipment. Both signals, along with the scan pulse generated by the optical detector 46, are sent to the control electronics 36 for processing. In preferred embodiments, such electronics include separate circuit boards for processing the individual horizontal and vertical signals into a video signal, which is then

processed by a third circuit board prior to output to either the video camera 24 for real-time viewing, or to the videotape recorder 40 or frame-grabber 38 for permanent storage.

With reference to Fig. 2, the articulated arm 20 of the confocal microscope allows the imaging field 14' to be effectively delivered to the sample for imaging purposes. Reflecting elbow-joint mirrors 68 allow the arm to be easily translated and manipulated with respect to the sample, thereby facilitating real-time *in vivo* imaging of tissue which may lie in hard-to-reach places. In addition, because the arm is mobile and thus follows the movements of the subject, small time-dependent fluctuations in the tissue, such as those corresponding to the subject's breathing or heart beat, can be corrected when the arm is secured to the subject. Although a single joint is shown in the arm of the figure, it is understood that multiple joints may be included in order to lend flexibility and additional mobility. Generally, joints with 90° angle turns for each joint are used. Alternatively, other joint angles may be used. This design offer improvements over conventional confocal microscopes, wherein such fluctuations occurring in live subjects may move the image plane out of focus, thereby reducing the quality of the image.

The galvanometric mirror 56 is positioned at the pivot point of the arm, and, as described previously, is positioned at the conjugate image plane corresponding to the object plane of the polygon mirror so that movement of the field on the mirror's surface is minimized. During operation, the mirror 56 undergoes two types of angular motion: rapid, oscillatory angular motion (at the frequency, e.g., of 60 Hz) allows the beam to be scanned along the vertical (y) dimension for imaging purposes,

while slow, non-oscillatory motion allows the imaging field 14' to be centered on the mirror 68 during movement of the arm. As described above, the rapid oscillatory scanning is driven at a standard video rate (e.g., 60 Hz), resulting in the beam being continuously displaced by distances of a few millimeters along the raster plane 70. In order to minimize spatial wander, the aperture of the microscope objective lens 72 is positioned at the conjugate image plane corresponding to the object plane 64 of the galvanometric mirror. The mirror 56 is rotated in a slow, non-oscillatory fashion when the arm 20 is displaced, as indicated in the figure by the arrow 65. In this manner, the galvanometric mirror 56 may be rotated in a compensatory manner by angular deviations ranging from a few tenths of a degree to several tens of degrees to allow continuous centering of the beam on the mirror surface. Typically, this slow motion is accomplished with a mechanical gearing apparatus, although electrically induced motion, such as galvanometric motion, may also be used.

With reference now to Figs. 3A-3C, in order to image a section of tissue, the imaging field 14' incident on the tissue sample at the focal plane is scanned along the x and y dimensions as described above. Using the rotating polygon mirror (x axis scan; 15.7 kHz) and the oscillating galvanometric mirror (y axis scan; 60 Hz), the pattern of the field 14 preferably resembles a series of closely spaced raster lines 90, each having a width of about 1-50 microns when focussed onto the image plane. Preferably, each field includes 525 raster lines in order to conform to the United States television and video standard. In order to maximize the resolution of the image, neighboring raster lines 90, 91 are kept as close together as possible. These lines may touch each other along their common edge 93, as shown in Fig. 3B, or

overlap in portions with each other, as shown in Fig. 3C. Angular deviations of the scanning optics are preferably maintained at levels which allow the field 14' to irradiate the entire region to be imaged. In the image plane, for example, the field typically has x and y dimensions of between 50 and 250 microns, and most preferably between 100 and 200 microns. During imaging of skin, for example, this allows the operator to monitor properties of cells separated by distances of a few tens microns which have diameters on the order of a few of microns; cells present in all layers of the skin, e.g., the stratum corneum, epidermis, and dermis, can be imaged in this manner.

Referring now to Figs. 4A-4C, the scanning optics of the microscope can be manipulated to allow imaging of horizontal optical sections 100 at various depths along the z axis (as shown in Fig. 4A), or, alternatively, sections which can be continuously rotated about the x (Fig. 4B) and y (Fig. 4C) axes. Imaging in this fashion allows various components of tissue to be observed *in vivo* along a number of vantage points, thereby providing the operator with a method of performing a non-invasive, real-time alternative to a tissue biopsy.

Different sections are imaged by changing the scanning magnitude (i.e., the magnitude of the scanning angle or longitudinal displacement) of one or more of the scanning optics. For example, in the depth-dependent horizontal sections 100 shown in Fig. 4A, the x and y axes are scanned, while the z axis is translated incrementally. Different horizontal sections are imaged by gradually adjusting the distance separating the microscope objective lens 72 and the sample 22, with the depth of imaging ultimately being limited by the distance that light of a particular wavelength can propagate into the tissue. The separation distance can be adjusted

using, for example, a mechanical translational stage adjusted by hand or with a motor.

Sections rotated about the x or y axes are imaged by continuously scanning the imaging field about the z axis, and adjusting the relative horizontal or vertical scanning amplitudes. For example, as shown in Fig. 4B, an imaged section 111 lying in the xy plane can be rotated by continuously scanning along the z axis, and adjusting the vertical (i.e., y axis) scanning amplitude. In this case, such an adjustment can be made using a balance control, such as a simple potentiometer circuit, which allows the relative amplitudes of the waveforms sent to the driver which translated the scanning platform (17 in Fig. 1) and galvanometer driver (58) to be varied. The vertical section 110 represents the extreme example of this type of adjustment; here, the imaging plane has no component along the y axis, and is composed solely of the xz plane. Alternatively, as shown in Fig. 4C, the imaged sections may be rotated continuously from the horizontal section 121 positioned in the xy plane to the vertical section 120 positioned in the yz plane. In this case, rotation about the y axis may be carried out by adjusting the relative scanning amplitudes along the x and z axes. This may be done, for example, by inserting an additional galvanometric mirror into the imaging system shown in Fig. 1, or, alternatively, by replacing the rotating polygon mirror with a galvanometric mirror similar to that used for scanning along the y axis. Other methods for adjusting the horizontal scanning amplitude, such as replacing the rotating polygon mirror with a similar device having an adjustable number of facets, may also be used.

Once a continuously varying series of sections are generated, three-dimensional images may be constructed using standard image-manipulation techniques known in the

imaging arts. This allows, for example, tumors or other lesions near to the tissue surface to be diagnosed without using a biopsy.

The optical wavelength of the light source used in the confocal microscope is chosen to optimize the image quality for a particular sample. The confocal image is preferably taken in remittance, and thus the wavelength of the imaging field is should be partially reflected by the imaged tissue. Just as importantly, the wavelength must not be too strongly absorbed or scattered by the tissue, as this attenuates the imaging field and strongly limits the imaging depth that can be achieved. In general, longer wavelengths, such as those in the infrared between the major water absorption bands, are preferred, as this light is weakly absorbed by most tissue. In particular, the preferred wavelengths for imaging are between 700 and 2000 nm; shorter, visible wavelengths are strongly absorbed or optically scattered by most tissue, while selected bands and wavelengths deeper in the infrared (i.e., 1940 nm and wavelengths greater than about 3 microns) are absorbed by water present in most tissue, and should be avoided. An especially preferred wavelength range is near 1600 nm. Preferred light sources include diode ($\lambda \approx$ 800-1000 nm), Ti:Sapphire and infra-red dye ($\lambda \approx$ 700-1000 nm), ruby (λ = 694 nm), and alexandrite (λ = 700 - 850 nm), Nd:YAG and Nd:YLF (λ = 1064 and 1053 nm) lasers.

Light sources producing visible radiation, such as ion or gas lasers, may be used according to the invention. Although light produced by these sources is often more strongly absorbed by tissue, it is useful for generating high-resolution images due to the fact that it can be focussed to smaller spot sizes compared to infrared radiation.

The confocal microscope may be used to generate, non-invasive, real-time, video-rate images of living tissue. Tissue can be imaged *in vivo* and at depths as large as 1-3 mm in the skin; no tissue biopsy or other invasive technique is required for obtaining a specimen for microscopy. For example, the confocal microscope can be used to image the eye, ear, cervix (e.g., the cervix uteri), or other body orifices, such as the oral, nasal, or anal cavity. The confocal microscope is particularly useful for imaging cells and tissue in living skin. In this case, the confocal microscope can be used to image the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, stratum basale, dermis, blood vessels, keratinocytes, merkel cells, tactile (merkel) discs, melanocytes langerhans cells and sensory neurons. Thus, the confocal microscope can be used to detect, diagnose or assist in the diagnosis of suspected skin disorders such as skin cancers, e.g., basal cell carcinomas, and squamous cell carcinomas. In this embodiment, a skin lesion (e.g., a macule, papule, nodule, plaque, vesicle, or bulla) or suspected skin lesion is imaged by the confocal microscope. During certain procedures, confocal microscope images of the lesion or suspected lesion may be compared with corresponding images of normal (i.e. control) skin from the same body location and depth. Images of the skin lesion or suspected skin lesion can assist the clinician in selecting a treatment modality, e.g., excision, electrodesiccation and curettage, cryosurgery, radiation therapy, or Moh's surgery (see Wilson, J.D. et al. (eds.) *Harrison's Principles of Internal Medicine 12th ed.* (1991).

In other embodiments, the confocal microscope can be used to detect, diagnose or assist in the diagnosis of other skin diseases, e.g., eczema, psoriasis, skin infections (bacterial or viral), including

immunologically mediated skin diseases such as allergies; drug reactions in the skin, and dermatitis. In addition, warts, moles or acne can be deeply imaged by the confocal microscope to evaluate appropriate therapies. The confocal microscope can also be used to image living tissue in the cervix (e.g., ovaries) in order to detect, diagnose or assist in the diagnosis of tumors. In yet another embodiment, the confocal microscope can be employed to image living tissue in animals (e.g., dogs, cats, or laboratory animal skin) to detect, diagnose or assist in the diagnosis of animal disease.

The confocal microscope is also a useful research tool for investigating (in patients or laboratory animals) the effect of cytokines and hormones on cells, e.g., the effect of erythropoietic hormone on blood cell number or density. Using the microscope, imaging can be used to evaluate white blood cell diapedesis, nerve cell growth (e.g, regeneration following nerve injury) and inflammatory reactions associated with drugs or transplantation.

Other Embodiments

The confocal microscope can be modified in several ways to improve its resultant images. For example, in one improvement, further stabilization of living skin can be achieved by more rigid microscope-to-skin coupling, e.g., by using an adhesive or suction to rigidly fix the skin to the articulated arm of the confocal microscope. Ideally, the stability of the skin will be limited only by the subject's pulse. As currently designed, the confocal microscope can tolerate a low-frequency (measured rate is approximately 1.25 Hz) axial modulation due to the subjects pulse, thereby limiting its effect on viewed or "grabbed" images. Imaging of other tissue can also be improved by more rigid microscope-to-tissue coupling.

Other improvements include the use of a pulsed laser in the confocal microscope. A pulsed laser has many advantages. For example, a pulsed laser provides an increased signal-to-noise ratio, particularly when used with well-known lock-in detection schemes. Second, a pulsed laser will provide superior imaging when light remitted from the skin (or other living tissue) is fluorescent light; a pulsed laser will minimize photobleaching. Another improvement includes the use of a higher-power laser in the confocal microscope. For example, a laser exceeding 1 watt can increase the laser beam power at the skin surface beyond 40 milliwatts, thereby increasing skin (or other tissue) penetration power.

In the near-infrared and infrared spectral region, the confocal microscope (operating in the reflectance-mode) will image remitted light most efficiently if the laser emits light at wavelengths approximating the spectral "valleys" of the near-infrared and infrared water absorption bands. For example, a laser emitting light at about 1600 nm will be an excellent light source for the confocal microscope with extremely deep tissue penetration power.

In another improvement, refractive index matching between the confocal microscope objective lens and a tissue, e.g., skin, can improve image quality. For example, fluids with different refractive indices can be used to maximize refractive index matching. Matching fluids which can be used include immersion oils, water, solutions comprising a carbohydrate or polymer, e.g., sucrose, and carbohydrate gels, e.g., sucrose gels.

In yet another improvement, the photodiode detector aperture size can be made between 50 to 800 microns in diameter, preferably between 100 to 200 microns in diameter. Such a detector aperture size

modification will improve contrast and imaging capability of the confocal microscope

Examples

The following are examples of imaging studies conducted on human patients using the confocal microscope described in detail above.

Confocal Microscope Design For Imaging Skin

The confocal microscope used for imaging human skin tissue included either an argon ion laser (λ = 488 or 514; model Innova 100, Coherent, Palo Alto, CA), krypton ion laser (λ = 647 nm; model CR-3000K, Coherent, Palo Alto, CA) or Ti:Sapphire laser (λ = 800 nm; model Cobra 2000/Titan CWBB, Schwartz Electro-Optics, Concord, MA). In all studies, the laser was operated in reflectance-mode. Horizontal scanning was accomplished with a rotating polygon mirror (model M225030XLIM with controller VFC2, Lincoln Laser, Phoenix, AZ), while vertical scanning involved the use of an oscillating galvanometric mirror (model G325DT, General Scanning, Watertown, MA). All intermediate optics in the scan system consisted of achromat lenses. The detector for video rate imaging was a silicon avalanche photodiode (model C39050E, EG&G Optoelectronics, Quebec, Canada) which exhibited high sensitivity in the wavelength range of λ = 600-2000 nm. The aperture in front of the image detector had a diameter of 50 μm.

The detector output was sent to a video monitor (Sony Trinitron) connected to either an S-VHS videotape recorder (Panasonic AG-7300) or an eight bits/pixel frame grabber (model Pixelpipeline PTP425, Perceptics, Knoxville, TN). The control electronics interfacing the detector and video monitor were built from well-known designs (see, e.g., Webb et al., Scanning Laser Ophthalmoscope, *IEEE Trans Biomed Eng*, BME-28;488-492).

An illumination power of about 40 milliwatts was used to image the human skin samples. To obtain high-resolution images, a 100X, 1.32 numerical aperture (NA), microscope objective lens (oil immersion type, Leica, Malvern, PA) was used for focussing light into the skin. In order to minimize reflection effects at the skin/air interface, the 100X microscope objective lens contacted the skin surface through immersion oil (refractive index = 1.52). The immersion oil reduced light loss in the illumination and detection paths, thus increasing image quality. With this configuration, the lateral resolution was approximately 1 μm, the axial resolution (tissue "section" thickness) was approximately 3 μm, and the field-of-view was approximately 150 μm by 150 μm at the skin.

In order to adjust the distance separating the microscope objective lens and the skin surface, the lens was mounted on a micrometer stage with a resolution of about 1μm. By axially translating the objective lens relative to the skin, it was possible to obtain a sequence of confocal sections, beginning at the stratum corneum and penetrating through the epidermis and papillary dermis. The images were videotaped while the objective was being translated, and then "grabbed" off the videotape with the frame grabber; each "grabbed" image was an integration of four frames. The grabbed images were enhanced in several steps by background subtraction, image size scaling with bilinear interpolation of the pixel values, linear 3X3 filtering, histogram clipping, and contrast adjustment.

The skin of a subject was kept immobile within 10 μm (i.e., four axial resolution elements) by placing the forearm in a mechanical fixture (e.g., a metal plate attached to the subject's skin with clamps). The mechanical fixture minimized gross skin motion, and

allowed high-quality images to be generated with distortion due to tissue movement attributed to the subject's heartbeat or breathing. The movement tolerance allowed the microscope to image blood flow in the skin.

Comparison of Confocal and Non-Confocal Imaging

After local intradermal anesthesia (2% lidocaine with epinephrine), skin biopsies (3 mm diameter punch) from three subjects were taken from the same sites imaged with the confocal microscope. The subjects had skin types II, IV, and VI. Each skin biopsy was fixed in 10% formalin and processed by H&E. Cell features measured from light photomicrographs of H&E sections were generally subject to errors from, e.g., tissue shrinkage, dehydration, embedding, and cell deformation during sectioning.

Confocal images were obtained as horizontal sections. These images were compared to standard "H&E sections", where H&E refers to the standard histological technique of sectioning tissue, staining the sectioned tissue with hematoxylin/eosin stain, followed by visualizing the stained and sectioned tissue by light microscopy. H&E sections, especially of the skin, are usually viewed in a vertical plane. For example, in Fig. 5, a schematic drawing of an H&E skin section (left side) is compared to horizontal optical sections obtained by the confocal microscope (right side). As can be seen from the figure, the stratum corneum (granular, spinous, and basal layers) of the viable epidermis, "peaks" of dermal papillae, capillary loops (blood flow) in the papillary dermis, can be imaged by the microscope, although here, the plane is perpendicular to that generally seen in histological tissue sections.

Referring now to Fig. 6A-6H, *in vivo* images of living human skin made with the confocal microscope show

the stratum corneum (Fig. 6A); this skin layer was imaged due to specular reflection caused by the expected refractive index mismatch between the microscope immersion oil and the skin. At 15-20 μm below the stratum corneum, the first large and sparsely located nuclei signified the first layer of viable epidermis, i.e., the granular layer (Fig. 6B). Further penetration from the superficial to the deeper epidermal layers showed that nuclei become small and more numerous. These nuclei were localized to the upper and lower spinous layers (Figs. 6C and 6D) extending 20-150 μm below the skin surface.

Below the spinous layers, clusters of bright cells were identified as basal cells on the "peaks" of dermal papillae (Figs. 6E and 6F) at the dermo-epidermal junction. Confocal microscope imaging deeper through the papillae showed basal cells on circular loci (Fig. 8G). These loci increased in size until neighboring loci (Fig. 8H) touched each other tangentially, indicating that the "valleys" of the rete ridges had been reached. The peak-to-valley modulation of the dermo-epidermal junction occurred between 50-150 μm below the skin top surface.

Referring now to Fig. 7, in the papillary dermis, the confocal microscope detected blood flow within capillary loops. The capillary lumens were 8±2 μm in diameter, and appeared dark. Circulating blood cells appeared as small disks (5-9 μm) or large ovals (9-18 μm). The concentration of the small disks was about 1.3-8.5 X 10^6 per mm^3. From their shape, size, and concentration, the cells were identified as erythrocytes and leukocytes. Passage of a single leukocyte through a capillary filled and distended the capillary lumen. Some very small disks (2-5 μm) could also be detected; these are platelets. Other features which could be detected with the confocal microscope included bright spiral- or

crescent-shaped substructures inside nuclei (nucleoli), collagen fibers surrounding the capillaries, sweat ducts spiralling down through the epidermis, and hair follicles.

Melanin, an epidermal chromophore (absorber of light) in the 600nm to near-infrared region of the spectrum, was found to be a naturally-occurring contrasting agent when used with the confocal microscope. For example, the keratinocyte cytoplasm was observed in darkly pigmented skin imaged brighter than the cytoplasm of lightly pigmented skin. Figs. 8A and 8B show a comparison between the images of basal cells from two subjects with skin types III (Fig. 8A; more melanin) and VI (Fig. 8B; less melanin). The images were unenhanced single-frame images, and are effective in emphasizing skin contrast. Across skin types I, II, III, IV, V and VI, image contrast increased in all layers of the epidermis (granular, spinous and basal layers) as the cell content of melanin increased.

Graphical analysis of unenhanced single frame images showed increasing image contrast with increasing cell content of melanin (Figure 11). Increased image contrast in the skin cells of a subject with type VI skin and vitiligo was also observed (Figure 12, panels a and b). In Figure 12, cytoplasm in depigmented areas of cells, could not be well visualized in the epidermis. However, adjacent pigmented skin imaged brightly with better contrast. Graphical analysis of unenhanced single frame images showed increasing image contrast of pigmented cells vs. depigmented cells.

The above-described imaging data illustrates the ability of the confocal microscope to image singly scattered remitted light. This was confirmed by observing that remitted light from a polarized laser beam could be completely blocked with a crossed polarizer.

Unlike proteins and membranes within the cytoplasm of a melanocyte or keratinocyte, melanin is a dense amorphous polymer. Recent studies of Sepia melanin granules found that the refractive index of the granules is approximately 1.7 near 600 nm, a value exceeding that of the surrounding cytoplasm. Mie scattering theory [Kerker, *The Scattering of Light*, Academic Press, New York, 1969] describes single scattering by spherical particles as a function of their size and refractive index relative to the surrounding medium. In general, light scattering is greatest when particle size is nearly equal to the wavelength, and varies with the square of the refractive index difference between the particles and the surrounding medium. Melanosomes containing melanin fit this description: melanosome size is nearly equal to optical wavelengths in the near-infrared region and their refractive index is significantly higher than that of the surrounding cytoplasm. Hence, melanin strongly backscatters light, thus serving as a useful naturally-occurring contrast agent.

Confocal microscope images taken as described herein compared favorably to images to the observed H&E sections. In particular, when H&E samples were sectioned horizontally, cell feature measurements were comparable to horizontal measurements obtained by the confocal microscope.

Bright substructures were observed within nuclei of the granular and spinous layers. The substructures were either spiral- or crescent-shaped regions of greater contrast within dark nuclei; these are nucleoli. Confocal microscope optical sectioning through nuclei showed that the spiral structures were in the center of the nucleus, while the crescent structures were against the inner portion of the nuclear membrane. In comparison, nucleoli generally appear by H&E as spherical

bodies within nuclei. This observation points to an important difference between confocal microscope imaging and imaging tissue prepared by H&E. The confocal microscope images living tissue; cells and organelles appear as they exist *in vivo*. However, cells and organelles prepared by H&E appear distorted by light microcopy; this is due to e.g., fixation, microtome sectioning, and tissue staining.

Referring now to Fig. 9, an image of skin made by the confocal microscope employing a Ti:Sapphire laser (800 nm) indicates the advantages of using an infrared light source. Here, cell nests in a compound nevus were imaged. Melanin contrast enhancement allowed superior imaging of these cells. Nevus cells imaged in living tissue were larger than basal cells and hence were easily differentiated from the basal cells. Morphological features from one compound nevus are listed in Table I, below.

TABLE I

Feature	Measurement
Nucleus size	7 ± 2 μm
Inter-nucleus spacing	13 ± 2 μm
Cell density	2-5 cells per nest
Nest size	23 ± 6 μm
Inter-nest spacing	28 ± 5 μm
Nest density	1250 - 2000 per mm^2

Other embodiments are within the scope of the following claims.

CLAIMS

1. A confocal microscope for generating a video-rate, real-time image of a sample, comprising:

an optical delivery system including a light source for generating an imaging field,

a horizontal scanning element comprising a motor-driven, movable reflecting component positioned to receive and then reflect the imaging field in a time-dependent manner, thereby generating an elongated horizontal imaging field along a horizontal axis,

a vertical scanning element comprising a motor-driven, reflecting component which angularly oscillates about an axis in an adjustable, time-dependent manner and is positioned to receive and then reflect the horizontal imaging field along a vertical axis, thereby generating a two-dimensional imaging field comprising both horizontal and vertical imaging fields,

an imaging lens configured to focus the two-dimensional imaging field to a focal plane in the sample, and then, following remission from the portions of the sample lying in said focal plane, receive the remitted optical image,

a focal-plane scanning element, positioned at any point along the imaging field's optical pathway, comprising an oscillating, motor-driven reflecting or transmitting optical component for spatially varying, in a time-dependent manner, the focal plane of the two-dimensional imaging field, and

detector means for converting the remitted optical image into a series of electrical signals, and image processing means for processing the signals to generate a real-time, video-rate image of the sample.

2. The confocal microscope of claim 1, wherein said focal-plane scanning element is mounted on an oscillating stage configured to translate with an

adjustable longitudinal displacement along the optical pathway.

3. The confocal microscope of claim 2, wherein said focal-plane scanning element is positioned to receive and then transmit the two-dimensional imaging field.

4. The confocal microscope of claim 3, wherein said focal-plane scanning element is a lens.

5. The confocal microscope of claim 1, further comprising at least one controller including a frequency source for selectively varying the magnitudes of the angular rotation and longitudinal displacement of, respectively, the vertical and focal-plane scanning elements.

6. The confocal microscope of claim 5, wherein said focal-plane scanning element and said vertical scanning element are driven by a common frequency source.

7. The confocal microscope of claim 6, wherein said focal-plane scanning element is driven at a frequency of between 25 and 100 Hz.

8. The confocal microscope of claim 1, further comprising a pivoting articulated arm for housing the vertical scanning element and the imaging lens, said arm comprising at least one reflective elbow joint configured to receive the two-dimensional imaging field from the vertical scanning element and then deliver the field to the imaging lens.

9. The confocal microscope of claim 8, wherein said vertical scanning element is positioned at a pivot point of said articulated arm.

10. The confocal microscope of claim 8, wherein said articulated arm further comprises a focussing lens positioned along the two-dimensional field's optical pathway between the vertical scanning element and the imaging lens, said focussing lens configured to image the two-dimensional field onto an entrance aperture of said imaging lens so that spatial displacements of the field are minimized at said aperture.

11. The confocal microscope of claim 1, wherein said light source is a laser emitting light in the infrared spectral region.

12. The confocal microscope of claim 11, wherein said laser is a Nd:YAG laser.

13. The confocal microscope of claim 11, wherein said laser emits light at a wavelength between 1550 and 1800 nm.

14. The confocal microscope of claim 11, wherein said laser is a semiconductor diode laser.

15. The confocal microscope of claim 11, wherein said laser emits light at wavelengths less than 1400 nm.

16. A confocal microscope for generating a video-rate, real-time image of a sample, comprising:

an optical delivery system including a light source for generating an imaging field,

a horizontal scanning element comprising a motor-driven, movable reflecting component positioned to receive and then reflect the imaging field in a time-dependent manner, thereby generating an elongated horizontal imaging field along a horizontal axis,

a vertical scanning element comprising a motor-driven, reflecting component which angularly oscillates about an axis in an adjustable, time-dependent manner and is positioned to receive and then reflect the horizontal imaging field along a vertical axis, thereby generating a two-dimensional imaging field comprising both horizontal and vertical imaging fields,

an imaging lens configured to focus the two-dimensional imaging field to a focal plane in the sample, and then, following remission from portions of the sample lying in said focal plane, receive the remitted optical image,

a pivoting articulated arm for housing said vertical scanning element and said imaging lens, said arm comprising at least one reflective elbow joint configured to receive the two-dimensional imaging field from the vertical scanning element and then deliver the field to the imaging lens, and

detecting means for converting the remitted optical image into a series of electrical signals, and image processing means for processing the signals to generate a real-time, video-rate image of the sample.

17. The confocal microscope of claim 16, wherein said vertical scanning element is positioned at the pivot point of said articulated arm.

18. The confocal microscope of claim 16, wherein said articulated arm further comprises a focussing lens positioned along the two-dimensional field's optical

pathway between the vertical scanning element and the imaging lens, said focussing lens configured to image the two-dimensional field onto an entrance aperture of said imaging lens so that spatial displacements of the field are minimized at said aperture.

19. A confocal microscope for generating a video-rate, real-time image of a sample, comprising:

an optical delivery system including a light source for generating an imaging field,

a horizontal scanning element comprising a motor-driven, movable reflecting component positioned to receive and then reflect the imaging field in a time-dependent manner, thereby generating an elongated horizontal imaging field along a horizontal axis,

a vertical scanning element comprising a motor-driven, reflecting component which angularly oscillates about an axis in an adjustable, time-dependent angular manner and is positioned to receive and then reflect the horizontal imaging field along a vertical axis, thereby generating a two-dimensional imaging field comprising both horizontal and vertical imaging fields,

an imaging lens configured to focus the two-dimensional imaging field to a focal plane in the sample, and then, following remission from portions of the sample lying in said focal plane, receive the remitted optical image,

a pivoting articulated arm for housing said vertical scanning element and said imaging lens, said arm comprising at least one reflective elbow joint configured to receive the two-dimensional imaging field from the vertical scanning element and then deliver the field to the imaging lens,

a focal-plane scanning element, positioned at any point along the imaging field's optical pathway,

comprising an oscillating, motor-driven reflecting or transmitting optical component for spatially varying, in a time-dependent manner, the focal plane of the two-dimensional imaging field, and

detecting means for converting the remitted optical image into a series of electrical signals, and image processing means for processing the signals to generate a real-time, video-rate image of the sample.

20. A method of generating a video-rate, real-time *in vivo* image of tissue, said method comprising:

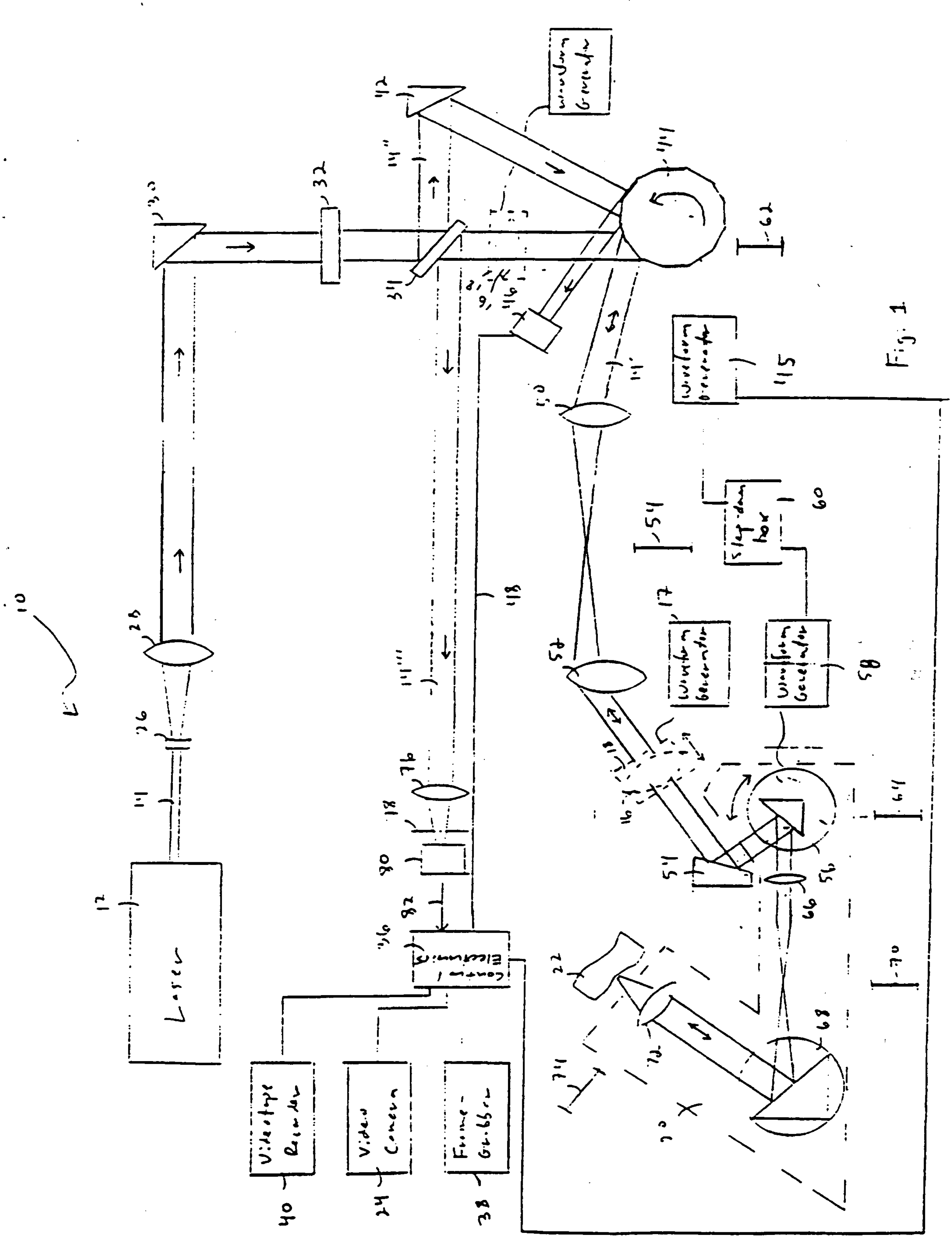
a) illuminating said tissue with an axially translated optical field, which optical field is focussed through a pivoting articulated arm attached to a microscope objective lens;
b) scanning said optical field through said tissue, which tissue remits light through said lens and said arm to a photodetector and a video monitor;

wherein, said photodetector and video monitor are operably linked, and together form a video-rate, real-time *in vivo* image of said tissue.

21. The method of claim 20, wherein said optical field is scanned in the x and y dimension.

22. The method of claim 20, wherein said tissue is human skin.

23. The method of claim 22, wherein melanin is used as a naturally occurring contrasting agent.


Laser
Video Camera
Videotape Recorder
Frame-Grabber
Fig. 1

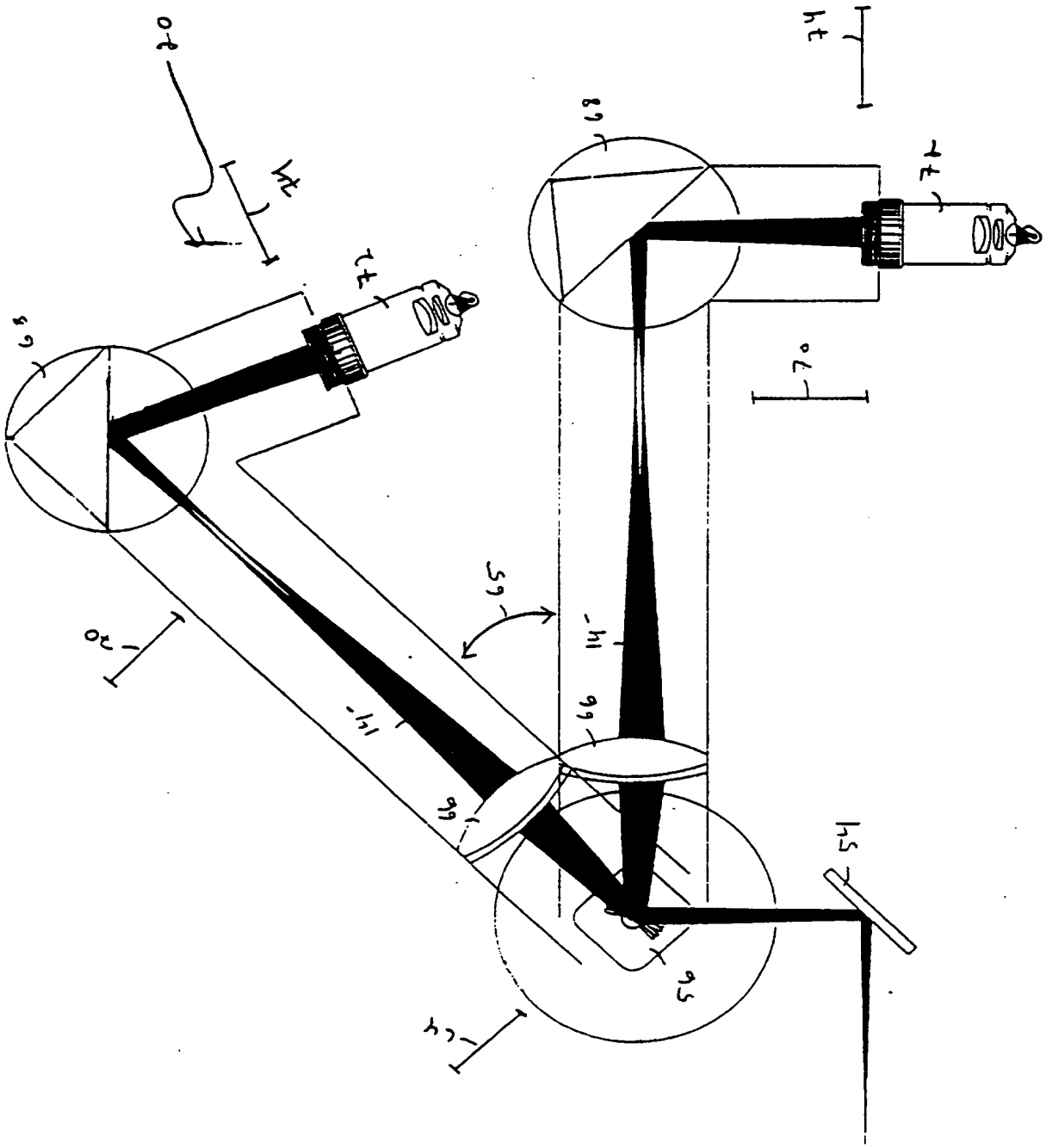


Fig 1

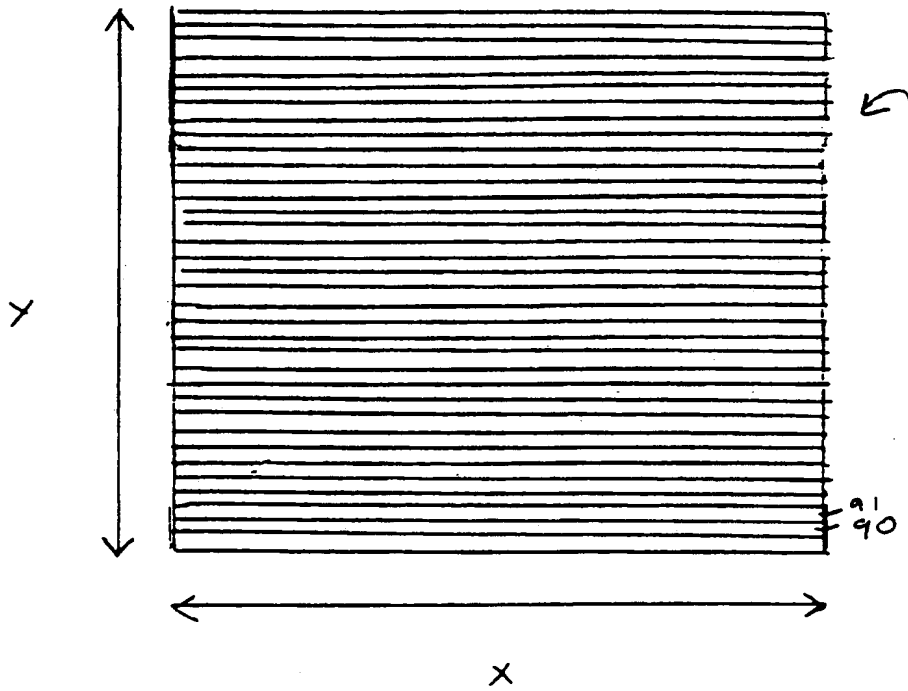


Fig. 3A

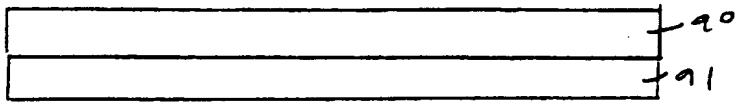


Fig. 3B

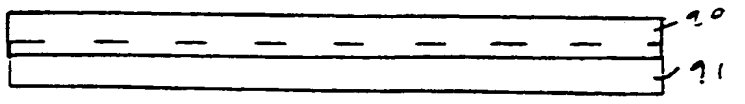


Fig. 3C

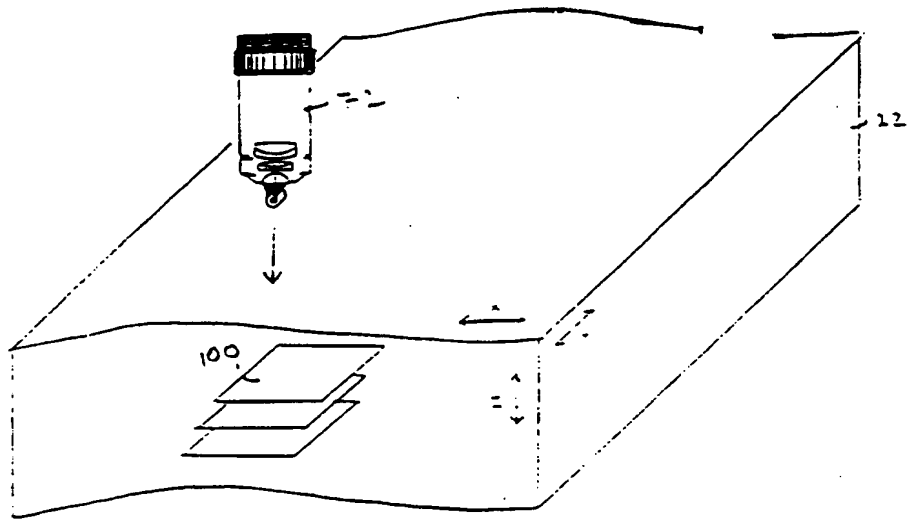


Fig. 4A

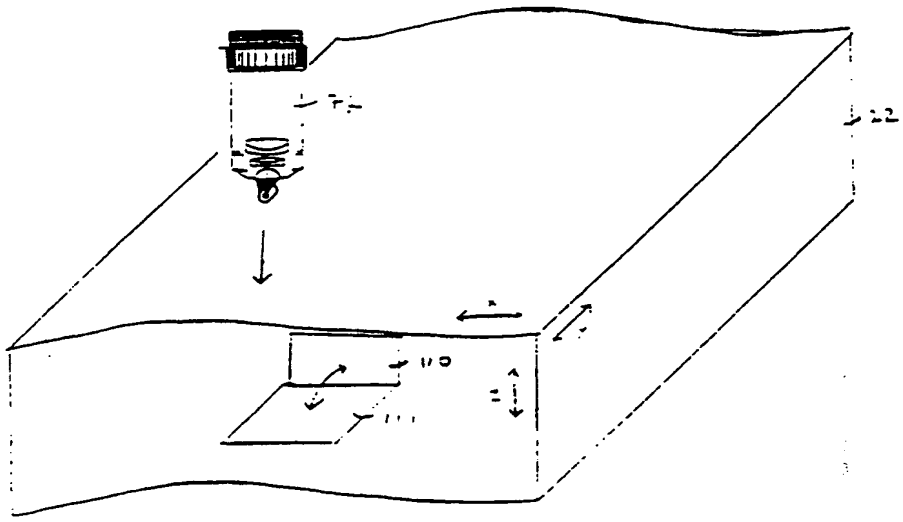


Fig. 4B

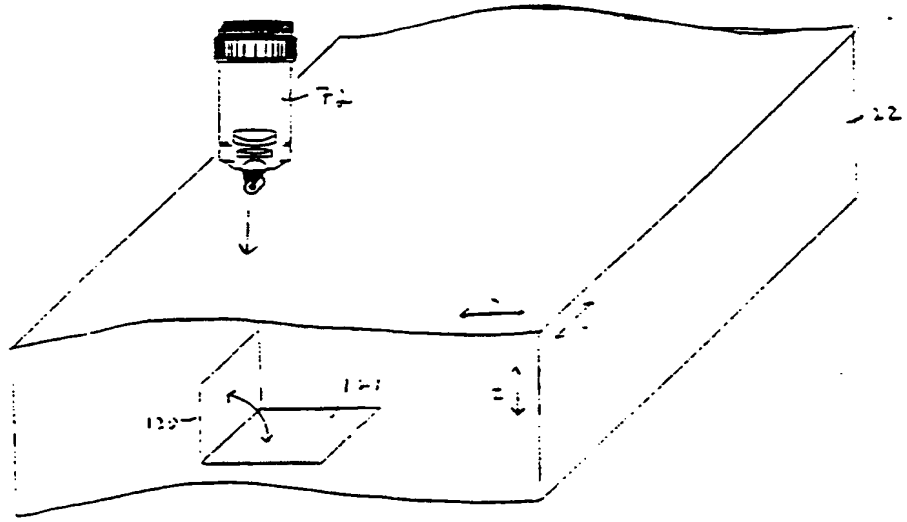


Fig. 4C

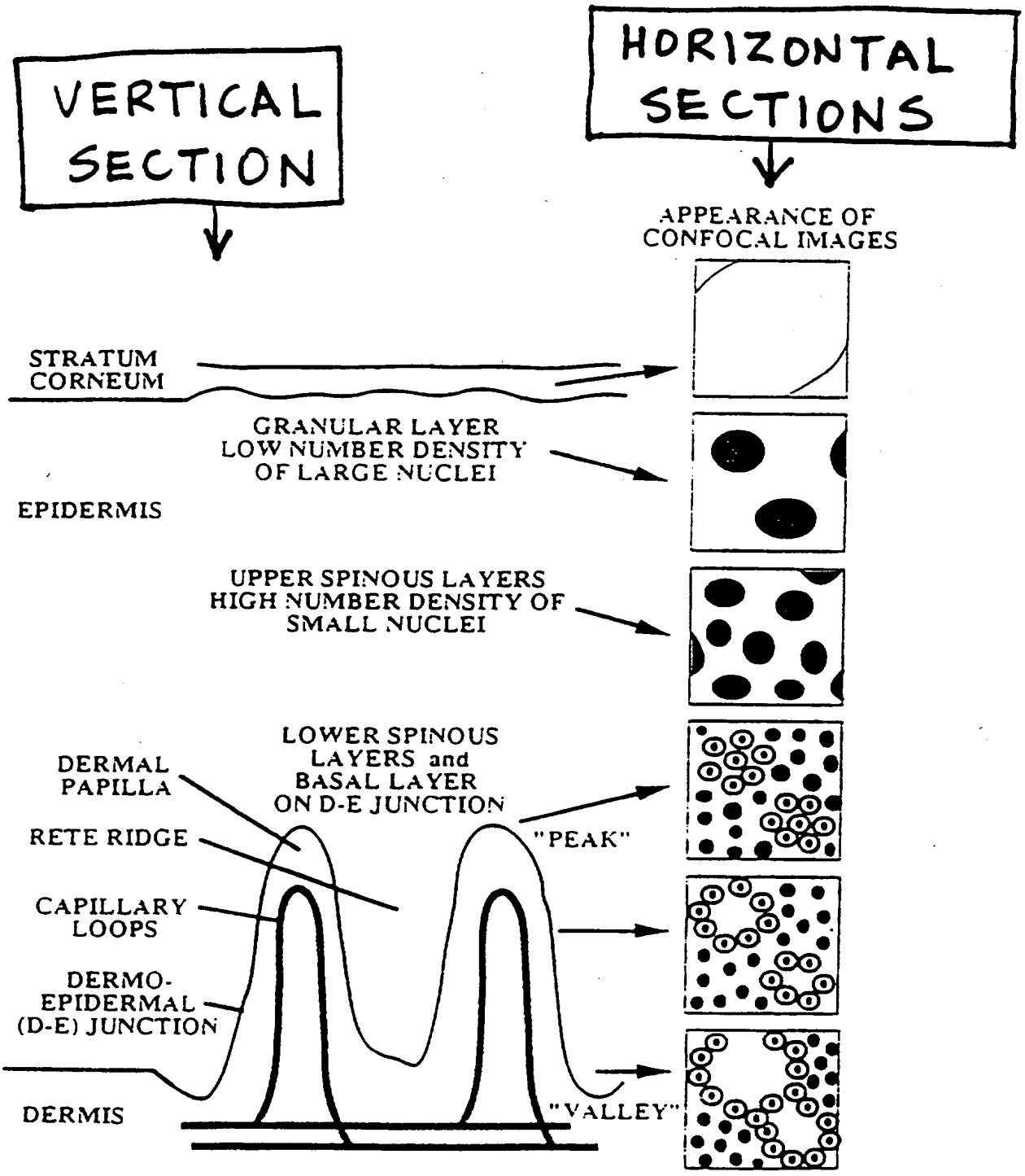
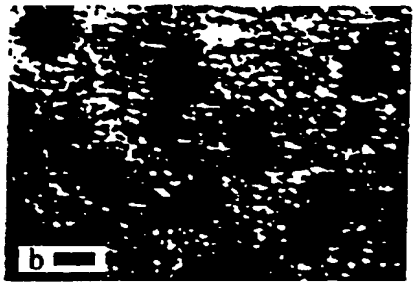

VERTICAL SECTION
HORIZONTAL SECTIONS
APPEARANCE OF CONFOCAL IMAGES
STRATUM CORNEUM
GRANULAR LAYER LOW NUMBER DENSITY OF LARGE NUCLEI
EPIDERMIS
UPPER SPINOUS LAYERS HIGH NUMBER DENSITY OF SMALL NUCLEI
LOWER SPINOUS LAYERS and BASAL LAYER ON D-E JUNCTION
DERMAL PAPILLA
RETE RIDGE
"PEAK"
CAPILLARY LOOPS
DERMO-EPIDERMAL (D-E) JUNCTION
DERMIS
"VALLEY"

Fig. 5

Fig. 6A

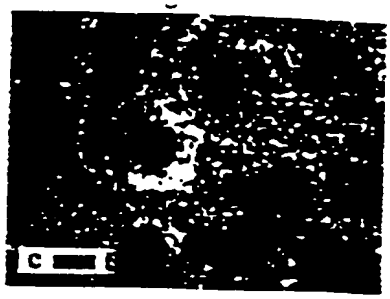
STRATUM CORNEUM

Fig. 6B



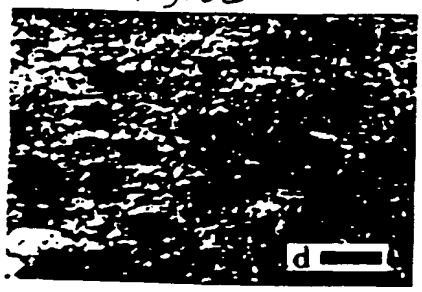
GRANULAR

Fig. 6C



UPPER SPINOUS

Fig. 6d



LOWER SPINOUS

Fig. 6e

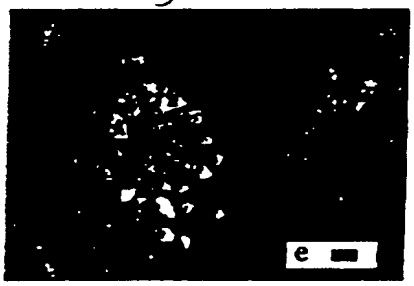


Fig. 6f

BASAL CELLS ON DERMAL PAPILLAE

Fig. 6g

Fig. 6h

BASAL CELLS ON DERMAL PAPILLAE

Fig. 7A

Fig. 7B

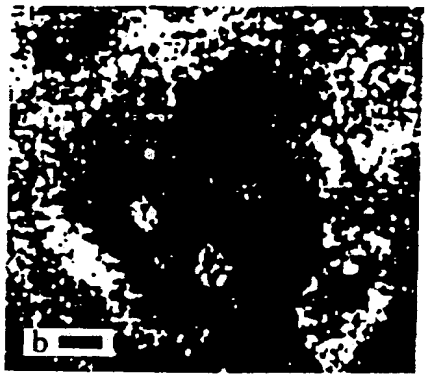
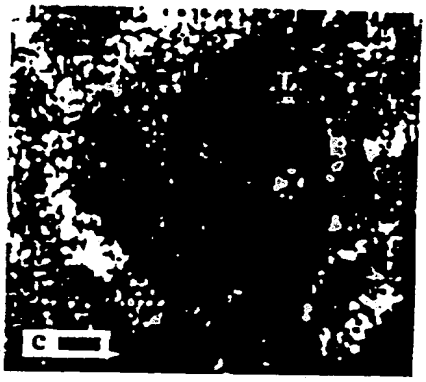


Fig. 7C



BLOOD FLOW (ERYTHROCYTES) IN PAPILLARY DERMIS

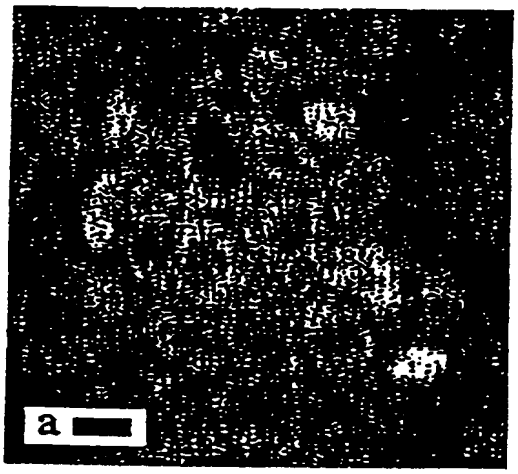


Fig. 8A

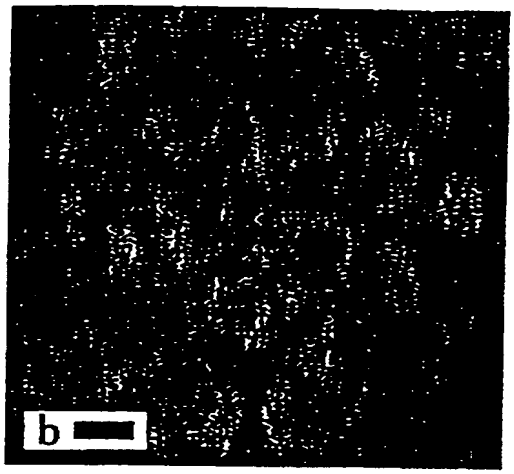


Fig. 8B

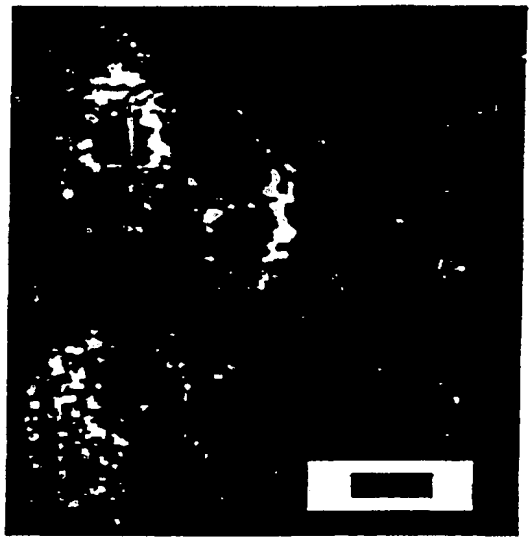


Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00448

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : H01J 3/14
US CL :359/368,369,372,380;250/216,234,236;358/79
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 359/363,368,369,372,380;250/216,234,236;348/79-80

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS for U.S. Patents

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A, 5,120,953 (HARRIS) 09 JUNE 1992, FIG. 1	1-5,11-15
Y	US,A, 4,959,552 (SAFFERT ET AL) 25 SEPTEMBER 1990, FIG. 3	1-5,11-15
Y	US,A, 4,834,516 (KAJITANI ET AL) 30 MAY 1989, COL. 5, LINES 25-30.	1-5,11-15
Y	US,A, 4,215,271 (MUDGE ET AL) 29 JULY 1990, COL. 3, LINES 28-33.	8-10,16-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
13 MARCH 1996	29 MAR 1996
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